Antiprimer Quenching-Based Real-Time PCR and Its Application to the Analysis of Clinical Cancer Samples

Jin Li, Fengfei Wang, Harvey Mamon, Matthew H. Kulke, Lyndsay Harris, Elizabeth Maher, Lilin Wang, and G. Mike Makrigiorgos

Background: Nucleic acid amplification plays an increasingly important role in genetic analysis of clinical samples, medical diagnostics, and drug discovery. We present a novel quantitative PCR technology that combines the advantages of existing methods and allows versatile and flexible nucleic acid target quantification in clinical samples of widely different origin and quality.

Methods: We modified one of the 2 PCR primers by use of an oligonucleotide “tail” fluorescently labeled at the 5’ end. An oligonucleotide complementary to this tail, carrying a 3’ quenching molecule (antiprimer), was included in the reaction along with 2 primers. After primer extension, the reaction temperature was lowered such that the antiprimer hybridizes and quenches the fluorescence of the free primer but not the fluorescence of the double-stranded PCR product. The latter provides real-time fluorescent product quantification. This antiprimer-based quantitative real-time PCR method (aQRT-PCR) was used to amplify and quantify minute amounts of input DNA for genes important to cancer.

Results: Simplex and multiplex aQRT-PCR demonstrated linear correlation ($r^2 > 0.995$) down to a DNA input equivalent to 20 cells. Multiplex aQRT-PCR reliably identified the HER-2 gene in microdissected breast cancer samples; in formalin-fixed, paraffin-embedded specimens; and in plasma circulating DNA from cancer patients. Adaptation to multiplex single-nucleotide polymorphism detection via allele-specific aQRT-PCR allowed correct identification of apolipoprotein B polymorphisms in 51 of 51 human specimens.

Conclusion: The simplicity, versatility, reliability, and low cost of aQRT-PCR make it suitable for genetic analysis of clinical specimens.

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cation or the formation of primer–dimers. Optimal primer design is therefore required in the latter case.

We present a novel method for signal generation in real-time PCR that uses fluorescently labeled PCR primers in combination with a universal quenching oligonucleotide (antiprimer) and combines many of the advantages of currently used approaches. The principle of multiplex antiprimer-based quantitative real-time PCR (aQRT-PCR)3 is illustrated in Fig. 1 (the primers and antiprimers used in aQRT-PCR are depicted in more detail in Fig. 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue4). One or more sets of primers are designed such that they carry a universal, 5′-positioned oligonucleotide “tail” fluorescently labeled with a different fluorophore for each target gene. An antiprimer carrying a molecule [3′-black hole quencher-2’ (3′-BHQ2)] that quenches all fluorescent probes simultaneously is also included in the PCR reaction. The antiprimer has a lower melting temperature ($T_m$) than the primer and is complementary to the primer tail. PCR product is synthesized over the primer tail after the second PCR cycle (Fig. 1), yielding 5′-end-labeled fluorescent double-stranded DNA products. After polymerase synthesis at a temperature suitable for the primer $T_m$, the reaction temperature is lowered by 5–10 °C to allow the antiprimer to bind and quench the free primer, and the fluorescence of the PCR product is then recorded. The polymerase synthesis step thus is decoupled from the signal detection step. Because the quenching molecule can be placed at any position along the antiprimer, the present approach allows the flexibility to achieve ideal quenching of the fluorescent primer, which enables strong signal generation. Below we demonstrate that this new real-time PCR methodology...

3 Nonstandard abbreviations: aQRT-PCR, antiprimer-based quantitative real-time PCR; BHQ2, black hole quencher-2; $T_m$, melting temperature; SNP, single-nucleotide polymorphism; FFPE, formalin-fixed, paraffin-embedded (specimens); FAM, 6-carboxyfluorescein; ROX, 6-carboxy-X-rhodamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; and Ct, threshold cycle.

Fig. 1. Outline of aQRT-PCR.

After the initial PCR cycling with fluorescently labeled primers, the temperature of the reaction is decreased to allow a quencher-labeled oligonucleotide (antiprimer) to bind the unincorporated primer. The antiprimer cannot bind to the double-stranded PCR product, which generates an exponentially increasing signal. Signal can be simplex or multiplex (also see Fig. 1 in the online Data Supplement).
offers high sensitivity, reproducibility, and multiplexing capability for detecting both gene amplifications and single-nucleotide polymorphisms (SNPs). The cost for aQRT-PCR is lower than for most current approaches, and this approach offers several advantages in the analysis of clinical samples. In validating the new method, we also developed a multiplex, single-tube methodology, using aQRT-PCR, to detect HER-2 copy number variations in microdissected breast cancer samples; in formalin-fixed, paraffin-embedded (FFPE) specimens; and in plasma circulating DNA.

**Materials and Methods**

**SOURCE AND EXTRACTION OF GENOMIC DNA**

Reference human male genomic DNA was purchased from Promega. BT474 genomic DNA was purified from a cultured BT474 breast cancer cell line obtained from the American Tissue Culture Collection. The 51 human surgical lung tissue samples were obtained from the Massachusetts General Hospital Tumor Bank, and the 4 Her2 breast cancer samples were obtained from the Dana Farber Cancer Center SPORE Bank after manual microdissection. The FFPE specimens were obtained from the Department of Pathology, Brigham and Women’s Hospital. Plasma samples were obtained from the Medical Oncology Tumor Bank, Dana Farber Cancer Institute. The use of unidentifiable human specimens for genetic analysis was approved by the Institutional Review Board. Genomic DNA from the BT474 cell line and from fresh tissues was extracted and purified by use of the DNAeasy Kit (Qiagen). A modified method was used for extracting DNA from FFPE specimens. Briefly, 25 mg of tissue per sample was deparaffinized by treatment with mixed xylenes. Deparaffinization involved mixing the sample with 1.2 mL of xylenes, vortex-mixing, centrifuging the mixture for 3 min at room temperature, and removing the xylene layer; this procedure was repeated 1 or 2 times until the xylene was clear. After deparaffinization, the xyles were removed by the addition of absolute ethanol (1.2 mL), vortex-mixing, centrifugation for 3 min at room temperature, and removal of the ethanol layer; this procedure was repeated 1–2 times until the ethanol layer was clear. After vaporization of the ethanol by incubation for 10 min at 37 °C, samples were washed in phosphate-buffered saline (addition of 1.2 mL of phosphate-buffered saline, vortex-mixing, centrifugation for 3 min at room temperature). After removal of the phosphate-buffered saline, the tissue was placed in 360 μL of lysis buffer (Qiagen) + 40 μL of proteinase K (concentrated) and rotated at 55 °C for 24–72 h as needed for full digestion. Subsequent DNA purification was carried out with the DNAeasy Kit, adjusting buffer and extraction volumes for the volume of lysis buffer used. The quality of the extracted DNA was initially evaluated by gel electrophoresis of 0.75 μg of DNA in a 1% agarose gel. To extract plasma circulating DNA, within 2–5 h of collection, whole blood was centrifuged at 2000g for 15–30 min, and the plasma was carefully collected from the top of the supernatant, as described previously (12). Plasma circulating DNA was purified from plasma with QIAmp MinElute Virus Spin Kit (Qiagen) and quantified by the PicoGreen method (Molecular Probes).

**SINGLE-GENE (SIMPLEX) aQRT-PCR**

For simplex aQRT-PCR, amplification was performed with the AmpliTaq Gold amplification reagents (Applied Biosystems) in a Smart-Cycler real-time thermocycler (Cepheid). The fluorescently labeled primer and nonlabeled primers (Table 1) were designed with Oligo 6 software (Molecular Biology Insights) and synthesized at Integrated DNA Technologies. 6-Carboxyfluorescein (FAM) and 6-carboxy-X-rhodamine (ROX) N-hydroxysuccinimide ester were used as labels at the 5’ end of the forward or, alternatively, the reverse fluorescence primer. Serial dilutions of DNA (0.14–145 ng) in a 1-μL volume were added to a final volume of 20 μL with a final concentration of 1× ABI TaqMan master mixture (Applied Biosystems). 0.2 μM each fluorescently labeled primer, unlabeled primer, and 1 μM

<table>
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<tr>
<th>Gene*</th>
<th>Forward</th>
<th>Reverse</th>
<th>Real-time PCR primers, 5’—3’</th>
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</thead>
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<tr>
<td>HER-2</td>
<td>GATGTTCCGCGGTCGTAAC</td>
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<tr>
<td>GAPDH</td>
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<td>ROX-GTGCTATCAGGAGCCCTGAGCTCGTACCTAAGAAA</td>
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<td>FAM-AGTGCTATCCGAGGGAATCCCTAGGAGA</td>
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</table>

* Fragment length in parentheses.

Table 1. Primers and probes for aQRT-PCR.
of genomic DNA to a final volume of 20 µL containing a final concentration of 1× ABI TaqMan master mixture, 0.05 µM each FAM-labeled HER-2 reverse primer and unlabeled HER-2 forward primer, 0.15 µM each ROX-labeled GAPDH forward primer and unlabeled GAPDH reverse primer, and 1 µM BHQ2-labeled antiprimer. The thermocycling program was 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 50 °C for 30 s, and 50 °C for 15 s (for reading fluorescence). Fluorescence was read in both the FAM and ROX channels simultaneously. Three independent experiments were performed for each gene to generate a mean (SD) relative copy number. The relative gene amplification ratio between unamplified/amplified plasma circulating DNA was calculated by the comparative threshold cycle (ΔΔCT) method (3, 13).

To examine clinical samples for HER-2 amplification by multiplex aQRT-PCR, we used 2 ng of genomic DNA from the microdissected breast cancer samples (Her2+) in the reaction. For the FFPE samples, we used 20 ng as input genomic DNA, and for the plasma circulating DNA samples, we used 1 µL from each Qiagen-purified DNA sample in the reaction.

TaqMan real-time PCR
We also performed TaqMan real-time PCR for HER-2 to compare with the results obtained by aQRT-PCR. The assay was done as described previously (13, 14). Briefly, amplification was performed with AmpliTaq Gold (Applied Biosystems) in a Smart-Cycler. Primers and probes for exonic regions of genes studied were synthesized by Biosearch Technologies. For each reaction, we added 2 ng of genomic DNA to a final volume of 20 µL with final concentrations of 1× ABI TaqMan master mixture (Applied Biosciences), 0.2 µM each primer, and 0.1 µM probe. The thermocycling program was as follows: 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min, 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Three independent experiments were performed for each gene to generate a mean (SD) relative copy number by the ΔΔCT method.

Allele-specific aQRT-PCR of B71 SNP
We adapted the published allele-specific PCR primers for the B71 SNP (C>T) of human apolipoprotein B (15) by adding different fluorescent probes to the tails of each allele. The ROX-labeled forward primer, specific for the C genotype (indicated by the italic font), was 5’-ROX-AGT-GCTATCCAGGGAAGAGACCAGCCAGTGCAC-3’, the FAM-labeled forward primer, specific for the T genotype, was 5’-FAM-AGTGTATCCAGGGAAGAGACCAGCCAGTGC-3’, and the reverse primer was 5’-CAAGGCTTGGCTCAGGGT-3’. The PCR reaction was conducted in a 20-µL volume using a Smart-Cycler real-time PCR instrument. The real-time PCR reaction was set up as follows: 40 ng of human genomic DNA from clinical lung samples, 0.2 µM each C- or T-genotype-specific forward primer; 0.2 µM reverse primer; 1× Stoffel polymerase buffer; an extra 30 mM KCl to a final concentration of 40 mM; 2 mM MgCl2; 50 µM each of dATP, dCTP, dGTP, and dTTP; 50 nL/µL dimethyl sulfoxide; 25 nL/µL glycerol; and 2 U of Stoffel Taq polymerase (Perkin-Elmer). The thermocycling program was 1 cycle of 95 °C for 2 min and 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 50 °C for 30 s, and 50 °C for 15 s (for simultaneous reading of fluorescence from the FAM and ROX channels). For multiplex genotyping, AmpliTaq Gold (Applied Biosystems) was used instead of the Stoffel Taq polymerase. To genotype the B71 SNP, we performed multiplex real-time PCR in a final volume of 20 µL with the following final concentrations: 1× ABI TaqMan master mixture (Applied Biosystems), 40 ng of genomic DNA, 0.05 µM FAM-labeled T-specific primer, 0.15 µM ROX-labeled C-specific primer, 0.2 µM unlabeled reverse primer, and 1 µM BHQ2-labeled antiprimer. The thermocycling program was 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 50 °C for 30 s, and 50 °C for 15 s (for reading fluorescence from the FAM and ROX channels). To determine the reproducibility of the multiplex aQRT-PCR approach for genotype determination, we repeated the experiments 10 separate times.

Multiplex PCR for Genotyping the B71 SNP in Clinical Samples
We performed multiplex real-time PCR to genotype the apolipoprotein B (B71) SNP in 51 clinical lung samples, using the same PCR protocol with 40 ng of genomic DNA as starting material.

To verify the results of the aQRT-PCR multiplex genotyping, direct PCR sequencing on all 51 samples was performed by Polymorphic DNA Technologies, Inc. (Alameda, CA) and by the Dana Farber Molecular Biology Core Facility.

Results
Design of fluorescently labeled primers and antiprimer
aQRT-PCR is designed to enable real-time PCR in simplex or multiplex formats for gene copy number determination and SNP genotyping using primers containing universal fluorescently labeled oligonucleotide tails (see Fig. 1 in the online Data Supplement). The tail consists of a 17-base
oligonucleotide added to the 5' end of the forward or, alternatively, the reverse gene-specific primer, depending on which placement is less likely to contain secondary (hairpin) structures, as predicted by Oligo 6 software. In the current applications, the Tm of this oligonucleotide tail is ~57 °C, as calculated by Oligo 6 software. The antiprimer complementary to this tail also has a Tm of ~57 °C, whereas the gene-specific portion of the primer is designed to have a higher Tm (~65 °C). At an annealing-extension PCR temperature of 60 °C, the primers can bind the target and induce polymerization without much interference from the lower-Tm antiprimer. When the temperature is subsequently decreased to 50 °C, the antiprimer binds the tail of the free, single-stranded primer, but not the double-stranded PCR product (Fig. 1). Because the antiprimer concentration is 2- to 4-fold higher than that of the primer concentration, the majority of the free primer is expected to bind the antiprimer at 50 °C, thus strongly quenching the primer fluorescence. Because the 5' end of the primer is opposite to the 3' end of the antiprimer, the interaction is mediated via exciton interaction (16, 17) (i.e., direct contact-quenching) between the 5'-fluorophore and the 3'-quencher present on the tail and antiprimer, respectively, which for most fluorophores provides stronger quenching than fluorescence resonance energy transfer (18). The quencher chosen here was BHQ2, which has a wide range of absorbance wavelengths; it is thus appropriate for quenching multiple fluorophores simultaneously, including the FAM and ROX used for multiplex PCR in the present work.

Careful design of primers by use of Oligo 6 minimizes the probability for secondary structures and primer–dimer formation in aQRT-PCR. Furthermore, by its design, the antiprimer does not participate in primer–dimer formation because the 3’-quenching molecule is also an effective polymerase block (4).

**SIMPLEX aQRT-PCR**

We used aQRT-PCR for simplex amplification and quantification of several genes from human genomic DNA (Table 1). Shown in Fig. 2A are primary amplification curves obtained with various amounts of starting genomic reference DNA, down to an equivalent of 20 cells, for the gene HER-2, and the calibration curve (log concentration vs Ct) is shown in Fig. 2B. For comparison, the TaqMan real-time PCR method was performed in parallel (Fig. 2, C and D). The 2 methods used the same PCR reagent set, annealing and extension temperatures, fluorophore and quencher, and essentially the same primers except that the forward primer used in aQRT-PCR also contains the 5’-FAM-labeled tail. Under essentially identical PCR conditions, aQRT-PCR generated significantly stronger fluorescence signals than did the TaqMan reaction (Fig. 2), which possibly reflects the stronger quenching effect produced by direct contact of FAM and BHQ2 in aQRT-PCR compared with the fluorescence energy transfer in TaqMan. The data also demonstrate that the 2 methods have similar Pearson correlation coefficients (r²), indicating their equivalency for simplex HER-2 quantification.

We tested 4 additional genes (TBP, MYC, HBEGF, and TOP1), which have different amplicon sizes (69–134 bp), by simplex aQRT-PCR performed in triplicate independent experiments. Representative primary amplification curves are depicted in Fig. 2 in the online Data Supplement. The results demonstrate strong signals and linear curves for log concentration vs threshold (r² >0.99), with a lower limit of input genomic DNA of ~20 cell equivalents (~0.1 ng of genomic DNA). The no-DNA controls (water) gave no detectable signals for at least 45–50 PCR cycles with the primers tested.

**MULTIPLEX aQRT-PCR**

We performed multiplex aQRT-PCR according to the scheme shown in Fig. 1, using FAM-labeled reverse and ROX-labeled forward primers for the HER-2 oncogene and the GAPDH housekeeping gene, respectively, to quantify HER-2 amplification in a single-tube reaction. Both fluorescently labeled primers have the same oligonucleotide tail as the simplex aQRT-PCR. For optimal coamplification of HER-2 and GAPDH, the ratio of FAM- and ROX-labeled primers was experimentally determined to be 1:3. Under these conditions, the 2 genes are amplified with similar amplification efficiencies when reference genomic DNA was used (Fig. 3A in the online Data Supplement). We next tested serial dilutions of the starting material, human male reference genomic DNA (0.14–145 ng); the multiplex aQRT-PCR response was linear on both channels simultaneously (panels B and C in Fig. 3 of the online Data Supplement). The multiplex assay was linear (r² ~0.995) down to an amount of starting material equivalent to 20 cells, whereas the negative control (water) was negative in both the FAM and ROX channels for at least 45 PCR cycles.

The ability of the multiplex assay to quantify, in a single reaction, the known amplification of the oncogene HER-2 in genomic DNA from BT474 breast cancer cells is shown in panels D and E in Fig. 3 of the online Data Supplement. The GAPDH-normalized threshold difference (ΔΔCt = 4.2) is in good agreement to that obtained with the simplex TaqMan assay, in 2 separate reactions (ΔΔCt = 3.9), for BT474 genomic DNA, as per our earlier report (14). Furthermore, in triplicate repeated experiments, a 20% dilution of BT474 DNA in reference DNA was reliably differentiated from pure reference DNA (curves 3–5 in panels D and E of Fig. 3 in the online Data Supplement). This indicates that the method should be able to detect a 20% minority of HER-2 amplified cancer cells in a background of 80% stromal cells.

We next examined the ΔΔCt for BT474 cells when the starting genomic DNA material was gradually decreased from 145 ng down to 0.14 ng. The results (Fig. 3F in the online Data Supplement) indicated that the ΔΔCt remains substantially constant even at low input DNA (relative standard deviation of the ΔΔCt, 13%), indicating the
ability of the multiplex approach to reliably quantify gene amplification in samples containing minute amounts of DNA, such as those obtained from fine-needle biopsies or from tissue microdissection.

HER-2 AMPLIFICATION IN CLINICAL SAMPLES DETECTED BY MULTIPLEX aQRT-PCR

HER-2 is overexpressed in 20%–30% of breast cancers (19), ovarian cancer (20), and other cancers (21, 22), and its expression is correlated with clinical outcome (19). To demonstrate the ability of multiplex aQRT-PCR to detect HER-2 amplification in fresh DNA from microdissected clinical samples, we tested HER-2 amplification in DNA extracted from 4 manually dissected breast cancer specimens characterized as HER2+/HER11001 by immunohistochemistry and fluorescence in situ hybridization in our institution (19). Shown in panels A and B of Fig. 3 are the primary amplification curves obtained with 2 ng of starting DNA (equivalent to ~350 cells) for the 4 samples and for reference and BT474 DNA by multiplex aQRT-PCR for HER-2/GAPDH. All 4 samples gave substantial (>8-fold) chromosomal HER-2 amplification, in agreement with the fluorescence in situ hybridization and immunohistochemistry results. The threshold difference (ΔΔCt) for the 4 samples ranged between 3 and 5 cycles, similar to the amplification detected in BT474 breast cancer cells.

To examine the utility of the method in situations in which the starting DNA material is of low quality and/or quantity, we applied multiplex aQRT-PCR to the detection of HER-2 in DNA from FFPE specimens as well as in free circulating DNA extracted from plasma of colon and ovarian cancer patients. DNA extracted from these clinical
samples is highly fragmented and is often difficult to amplify (12, 14, 23). As shown in panels C and D of Fig. 3, multiplex HER-2/GAPDH amplification using DNA from FFPE samples (20 ng each) obtained from glioma cancer patients revealed that the formalin fixation procedure caused considerable DNA degradation (Fig. 4 in the online Data Supplement; agarose gel images). Compared with the threshold obtained for the reference DNA (10 ng of DNA) in the same experiment, aQRT-PCR was not significantly affected by the fragmentation in the starting material. Multiplex HER-2/GAPDH amplification results for 4 plasma circulating DNA samples obtained from colon and ovarian cancer patients are shown in panels E and F of Fig. 3. In this case, 1 μL of purified DNA was used in each reaction, and 2 independent experiments were performed. One of the plasma samples obtained from a colon cancer patient (sample 4) harbored a 6-fold HER-2 amplification, whereas the remaining plasma samples were negative for amplification. The results indicate that for short amplicons, such as those used for
HER-2 and GAPDH (~70 bp each), multiplex aQRT-PCR is not significantly affected by fragmentation of the input material.

REAL-TIME SIMPLEX AND MULTIPLEX SNP GENOTYPING BY aQRT-PCR
To adapt aQRT-PCR for real-time SNP genotyping, we used allele-specific PCR based on a 3’-mismatched nucleotide (24, 25). We selected a well-studied polymorphism of the apolipoprotein B gene (B71; C>T) for validation of the method. The T-allele–specific primer was labeled with FAM, and the C-allele–specific primer was labeled with ROX; the reverse primer was unlabeled and was shared by the allele-specific primers. A common antiprimer was used to quench both allele-specific primers. Two DNA samples previously sequenced at the Dana Farber Core sequencing facility and known to be homozygous C/C and T/T were first tested by simplex aQRT-PCR using the Stoffel fragment of Taq polymerase. We found an 8-cycle threshold difference between the 2 alleles, corresponding to an ~256-fold difference in the fluorescence signal (Fig. 4A). We then applied the method in a multiplex format, using Stoffel Taq polymerase, but the
reaction yield was suboptimal (data not shown). We therefore used multiplex aQRT-PCR with the AmpliTaq Gold polymerase. For multiplex aQRT-PCR, the ratio of FAM- and ROX-labeled allele-specific primers yielding optimal signals for both alleles in our real-time PCR instrument was experimentally determined to be 1:3. The multiplex SNP genotyping results for 3 genomic DNA samples (40 ng each) containing the C/C (homozygous), C/T (heterozygous), and TT (homozygous) genotypes are shown in Fig. 4B. The genotypes of these 3 samples were verified by sequencing (Fig. 5 in the online Data Supplement). To examine the reproducibility of the multiplex approach, we repeated the experiment 9 more times; the mean (SD) ΔCt values are shown in Fig. 4C. The Ct values obtained differed by 4.5 (0.7) cycles (C/C) and 3.5 (0.4) cycle (T/T) between each of the 2 homozygous samples and the heterozygous (C/T) sample. Assuming Poisson statistics, the threshold range covered by (ΔCt ± 3 SD) is expected to cover 99.73% of the distribution of values (26). Accordingly, multiplex aQRT-PCR was able to differentiate the 3 apolipoprotein B genotypes with high confidence.

MULTIPLEX SNP GENOTYPING OF CLINICAL SAMPLES
BY aQRT-PCR
To further validate the use of multiplex aQRT-PCR in clinical samples, we used the method to determine the apolipoprotein B genotype in genomic DNA extracted from 51 surgical lung specimens. Duplicate independent multiplex aQRT-PCR experiments were carried out, and the mean ΔCt (ROX-FAM) was calculated. We determined genotype by comparing the ΔCt to the (ΔCt ± 3 SD) range of genotype-specific thresholds derived from the experiment shown in Fig. 4C. The DNA was sequenced in parallel. The results of this study indicated complete (51 of 51) agreement between the 2 independent methods (Table 2).

**Table 2. Comparison of genotyping results obtained by multiplex aQRT-PCR and by sequencing for 51 clinical lung samples.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Samples, n</th>
<th>Concordance</th>
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<tr>
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<tr>
<td>CT</td>
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<td>T</td>
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<td>Total</td>
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Discussion

We validated aQRT-PCR, a real-time PCR methodology that is a novel modification of principles originally described by Nazarenko et al. (7), Morrison et al. (27), and Li et al. (28). The new method uses primers containing a universal oligonucleotide tail labeled with a single fluorescent reporter molecule. In multiplex PCR applications, primers containing different fluorescent probes were used in conjunction with a single probe (antiprimer) carrying a 3' quencher. aQRT-PCR thus could be used to quantify a wide range of targets in simplex or multiplex reactions in conjunction with one-time synthesis of the antiprimer. By decoupling the probe carrying the fluorophore from the probe carrying the quencher, the current approach substantially reduces the overall cost relative to real-time PCR methods requiring double labeling of every gene-specific primer or probe (3–5, 8–11). As a result, aQRT-PCR lends itself to cost-effective adaptation for high-throughput applications.

In a comparison with the TaqMan assay for real-time quantification of the HER-2 gene, aQRT-PCR demonstrated similar sensitivity while providing stronger fluorescent signals attributable to the optimal (contact) quenching made possible between the fluorescent probe and quencher by the antiprimer design. Because the TaqMan assay uses an oligonucleotide probe that must bind the template for signal generation, the TaqMan assay is still expected, in principle, to provide higher target specificity than methods that use fluorescent primers, such as aQRT-PCR. On the other hand, signal generation in aQRT-PCR is not dependent on the 5’→3’ exonuclease action of the polymerase, thereby enabling use of a variety of polymerases, including improved, high-processivity, low-cost polymerases that lack this property. In addition, similar to all methods that use a probe for signal generation, multiplexing using the TaqMan assay is difficult because of the increased probability for primer–probe interactions during PCR. Thus, 2 separate reactions must be used for detection of HER-2 gene copy number changes in breast cancer samples (14). This may increase the uncertainty in target quantification for several reasons. For example, inherent factors that can lead to between-tube or between-sample variability are thermocycler-dependent temperature deviations, the presence of individual DNA polymerase inhibitors in clinical samples, pipetting variations, or the abundance of the target sequence in the specimen of interest (2).

In contrast, adaptation of aQRT-PCR to the multiplex quantification of clinically relevant targets was straightforward, as demonstrated by the ability of the assay to detect HER-2 oncogene amplification in clinical cancer specimens from diverse sources and input DNA quantities and qualities (microdissection, FFPE specimens, plasma). The single-tube format of multiplex aQRT-PCR makes this technique highly reproducible because between-sample variability is minimized. As a result, it is possible to reliably identify HER-2 amplification in the presence of up to 80% background DNA. Detection of HER-2 amplification in the presence of excess nontumor (stromal) cells may provide an alternative to the costly and laborious approach of characterizing surgical tissue specimens by fluorescent in situ hybridization (19).

Identification of SNPs by aQRT-PCR in clinical samples by use of allele-specific PCR is another useful adaptation of the technology that could find wide applications both in medicine and biotechnology. Detection of the apolipoprotein B B71 polymorphism in clinical samples in multiplex format was reliable and in agreement with sequencing in 51 of 51 cases (Table 2). The ability to perform multiplex SNP detection in a single tube is expected to decrease inherent PCR uncertainties and
minimize cost and labor, thus enabling the technology to potentially be used in SNP quantification in allele pools (29, 30), for rapid assessment of sample quality after DNA/RNA extraction from FFPE samples or bodily fluids (23), and in other applications.

In summary, we present a novel real-time PCR methodology, aQRT-PCR, which combines simplicity, versatility, and low cost. We demonstrated the applicability and reliability of the aQRT-PCR method to the analysis of clinical samples in diverse situations. We also demonstrated a new approach for the evaluation of HER-2 gene copy amplification in clinical cancer samples. We expect that aQRT-PCR will find a wide range of applications in medicine and biotechnology.

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